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Matriconditioning Improves Thermotolerance in Pepper Seeds through Increased in 1-Aminocyclopropane-1-Carboxylic Acid Synthesis and Utilization

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Matriconditioning using a solid carrier, Micro-Cel E, was applied on pepper seeds and its effects on the improvement of thermotolerance through the ethylene biosynthesis were studied. Untreated and matriconditioned seeds were soaked in 5 mM 1-aminocyclopropane-1-carboxylic acid (ACC) for various time at 25 °C to studies the time course of ACC-derived ethylene production. To study the performance of the seeds at high temperature, they were planted at temperature regime of 35 °C, 12h light/27 °C, 12h dark. The ACC-oxidase activity of the seeds after incubated in ACC at 35 °C was also determined. The ACC contents in untreated and matriconditioned seeds during the 24h imbibition period at 35 °C were monitored. No ethylene was detected during soaking of pepper seeds in the absence of ACC. In 5 mM ACC detectable levels of ethylene were produced; the matriconditioned seeds producing 10-fold larger amounts than the untreated seeds at the time of germination. It is postulated that matriconditioning greatly increases the ACC-oxidase activity at the time of germination. Matriconditioned seeds imbibed at 35 °C produced larger amounts of ACC and greater ACC-oxidase activity than the untreated seeds. Thus, the basis for the thermotolerance by matriconditioned seeds may be increasing ability to synthesize ACC and to utilize it for ethylene production and stress alleviation.

Key words: ACC-derived ethylene production, ACC-oxidase, germination, preplant conditioning

INTRODUCTION

Ethylene promotes germination of seeds of a number of plant species (Abeles & Lonski 1969; Ketring 1977; Logan & Stewart 1991). It plays an important role in seed germination, especially under stressful conditions (Khan & Huang 1988; Khan & Prusinski 1989; Khan & Andreoli 1992; Khan *et al*. 1994). Under no stressful conditions germination does not appear to be influenced by inhibition of ethylene biosynthesis (Khan *et al*. 1987; Khan & Huang 1988).

Ethylene is synthesized in plant tissues from methionine via S-adenosylmethionine (SAM) and ACC (Adams & Yang 1979; Yang & Hoffman 1984; Yang 1985). The conversion of SAM to ACC is catalyzed by ACC-synthase, the rate-limiting enzyme, in the cytosol (Satoh & Yang 1989). The last stage of ethylene biosynthesis is catalyzed by ACC-oxidase, which is labile; its activity is dependent upon the integrity of cell membranes and tissues (Porter *et al*. 1986; Kende 1989). The ACC-oxidase activity is usually determined *in situ* by measuring the capacity of tissues to convert the exogenously applied ACC into ethylene (Ievinsh *et al*. 1990).

Seed vigor is related to the ability of seeds to produce ethylene. The inability of poor quality or low vigor seeds to perform well, especially under stressful conditions, was found to be related to reduced ethylene production in rape (*Brassica napus*) (Takayanagi & Harrington 1971) and snap bean (*Phaseolus vulgaris*) seeds (Samimy & Taylor 1983). Many seeds produce barely detectable levels of ethylene at the time of germination (Samimy & Taylor 1983; Khan *et al*. 1987; Prusinski & Khan 1990; Gorecki *et al*. 1991). Because of the low level of ethylene produced by the seed, particularly as the vigor decreases, it has proved difficult to use endogenous ethylene production to score for low vigor seed classes. The ACC-derived ethylene production, which measures ACC-oxidase activity, has been found to be a useful and sensitive measure of seed vigor in rice (Khan & Seshu 1986), lettuce (Khan & Andreoli 1992; Khan 1994), cabbage, tomato, snap bean, and sweet corn (Khan 1994) seeds. Seeds in the presence of ACC produced a much larger amount of ethylene than those in the absence of ACC (Khan 1994).

The stress resulting from high temperature, osmotic restraint, anaerobiosis, and flooding appears to inhibit the ACC-to-ethylene conversion step in preference to the SAM-to-ACC step in ethylene biosynthesis (Bradford *et al*. 1982; Yang & Hoffman 1984; Corbineau *et al*. 1988; Khan & Huang 1988; Khan & Prusinski 1989; Khan & Andreoli 1992).

Preplant conditioning of seeds in low ψ solutions has been found to be highly effective in alleviating thermoinhibition in lettuce and other seeds (Khan 1992). Matriconditioning lettuce seeds with Micro-Cel E at 15 °C for 20h removed the thermoinhibition and allowed the seeds to germinate at 35 °C. Preconditioned seeds were shown to synthesize ACC at 35 °C and to convert it to ethylene. The increase in ACC level as well as the alleviation of thermoinhibition in preconditioned seeds was negated by the presence of AVG, and reversed by the addition of exogenous ACC (Huang & Khan 1992).

A study with lettuce seeds showed an increase in the level of ACC during conditioning and an increase in ACC-oxidase activity in conditioned seeds during relief of thermoinhibition (Khan & Andreoli 1992). The relief of thermoinhibition by matriconditioning, thus, appears to involve a build-up of ACC and its utilization for ethylene production.

The objectives of the studies were: (i) to determine if matriconditioned and untreated pepper seeds significant different in their ability to produce ethylene, (ii) to determine if thermotolerance is acquired by matriconditioning in pepper seeds, and (iii) determine the relationship of thermotolerance to ACC metabolism.

MATERIALS AND METHODS

Seeds Materials. 'El Paso' pepper seeds were obtained from PetoSeed Co, Inc., Saticoy, California, USA. Seeds were kept at 6 °C and 33% RH, and small batches were removed as needed.

Matriconditioning Procedure. The procedure for matriconditioning has been described by Khan *et al*. (1990; 1992) and Khan (1992). Seeds were matriconditioned in a mixture of 16 g seeds, 4.8 g Micro-Cel E and 16 ml water (containing 0.2% thiram) for 2 and 4d at 25 °C or a mixture of 16 g seeds, 4.8 g Micro-Cel E and 22 ml water (containing 0.2% thiram) for 4 and 7d at 15 °C in the light in loosely capped glass jars. Matriconditioned seeds were washed and dried by forced air at 25 °C for 2h before sowing in either petri plates or Cornell peat-lite mix soil, which consisted of peat, vermiculite, lime, and fertilizer (Khan *et al*. 1983).

Time Course of ACC-Derived Ethylene Production. Untreated or matriconditioned (4d at 25 °C) seeds (0.5 g per replicate, three replicates) were soaked in 5 cm petri plates with 3 ml of 5 mM ACC or water for various time at 25 °C under fluorescent light (9 μmol m^{-2} s^{-1}). At indicated times, seeds were rapidly wipe-dried on paper towels and transferred to a 5.7-ml glass tube, capped with a rubber septum and incubated on its side for 2h at 25 °C in light. Ethylene content in the gas phase of the tube was determined as described before (Khan & Prusinski 1989). One-ml gas samples were withdrawn with a gas-tight syringe and injected into a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a 183-x 0.32-cm stainless steel column containing Poropak Q. Germination (seeds with visible radicles, usually 1-2 mm in length) percentages of untreated and matriconditioned seeds were also recorded.

Thermoinhibition and ACC-Oxidase Activity. To study the performance of matriconditioned and untreated pepper seeds at high temperature, they were planted in growth chamber at 35 °C, 12h light/27 °C, 12h dark temperature regime. Emergence tests were carried out in plastic boxes 30 x 25 x 12 cm filled one-third with Cornell peat-lite mix. Twenty-five seeds were planted per row, six rows per box, and each treatment was replicated four times. Light in the growth chamber from fluorescent tubes and incandescent bulbs was set at approximately 140 μmol m^{-2} s^{-1}. Emergence was scored daily, and the T_{50} was computed as time (day) to reach 50% of final germination. Shoots from fifteen seedlings, taken from the middle of each row, per replication, were excised at the soil surface at the end of observation (after 14d) and the fresh weight determined.

For ACC-oxidase activity determination, the untreated and matriconditioned seeds (25 seeds per replicate, three replicates) were incubated in a 5.7-ml tube in the presence or absence of 0.2 ml 5 mM ACC for various times at 35 °C. Ethylene analysis was the same as described above. The activity of ACC-oxidase was calculated by subtracting the amount of ethylene produced without ACC from that produced with ACC (Ievinsh *et al*. 1990).

ACC Determination. For ACC determination, matriconditioned (4d at 25 °C) and untreated seeds (0.5 g per replicate, four replicates) were imbibed at 35 °C in light for up to 24h with 3 ml of deionized water in 5 cm petri plates (without filter paper). Seeds were homogenized at room temperature with 80% cold methanol using a mortar and pestle. The homogenate was centrifuged at 5000-x g for 20 minutes and the supernatant dried *in vacuo* at 45 °C. The ACC content in the dried extract was determined by chemical degradation of ACC to ethylene according to Lizada and Yang (1979). Briefly, the dried extract was suspended in 2 ml distilled water and kept on freezer. A 0.9 ml aliquot of the aqueous extract was transferred into a 5.7-ml glass tube and 0.1 ml of 1 μmol $HgCl_2$ pipetted into the tube. The tube was sealed with a rubber septum and kept on freezer. Approximately 0.1 ml of a cold mixture of 5.25% NaOCl and concentrated NaOH (2:1, v/v) was injected by means of a syringe into the test tube, which was stirred briefly and then incubated for 3 minutes on freezer. One-ml gas sample was removed and injected into the GC. The amount of ACC in the sample was calculated as the amount of ethylene released from the sample. The efficiency of ACC to ethylene conversion was found to be about 60% (Ievinsh *et al*. 1990). The amount of ACC leaked in the soaked medium in the petri plate was determined by pipetting the soak solution into a 25 ml Erlenmeyer flask. The petri plate was rinsed three times with small amount of 80% cold methanol and the washes combined with the soaked medium. The combined solution containing ACC was dried *in vacuo* and the ACC content was determined as above.

Data Analysis. Quantitative data on percentage germination, emergence, T_{50}, seedling fresh weight, and ethylene production were analyzed using analysis of variance (ANOVA) and LSD.

RESULTS

Differences in Ethylene Producing Ability of Untreated and Matriconditioned Pepper Seeds. The amount of endogenous ethylene produced by pepper seed is too low to be detected by gas chromatographic means, it was postulated that the addition of external ACC would enhance the level of detectability and permit a study of the differences in ethylene biosynthesis during imbibition and germination of untreated and matriconditioned pepper seeds.

To detect ACC level that would produce relatively high levels of ethylene during periods of soaking of pepper seeds without adversely affecting subsequent germination, different

concentrations of this chemical were used. Untreated pepper seeds produced a measureable amount of ethylene in 1 mM ACC after 24h of soaking at 25 °C (1.09 nl/h/100 seeds) and the amount increased up to 52h of soaking (3.17 nl/h/100 seeds) before germination has occurred, then declined afterwards (54h of soaking) coinciding with the start of germination (5%). When the seeds were soaked in 5 mM ACC the ethylene produced was detected at 12h of soaking (1.77 nl/h/100 seeds), the amount increased at 48h (5.63 nl/h/100 seeds) then it stayed relatively stable up to 52h (2% germination) before started to decline at 54h coincided with germination increase to 7.5%. No ethylene was detectable in the absence of ACC up to 72h of soaking even though germination (8%) has started (Table 1). A concentration of 5 mM (presumably a saturating dose) was finally selected for various studies as more ethylene was produced at 5 mM than at 1 mM ACC.

The percent of germination, the time course of ethylene production and the amounts of ethylene produced by the untreated and matriconditioned seeds varied a great deal (Figure 1). In untreated seeds, two ethylene peaks were found, one at 24h seed soak, prior to germination, and the other at 56h coinciding with the beginning of germination (Figure 1a). In seeds conditioned at 25 °C for 4d, a relatively smaller peak of ethylene at 6h was followed by a large increase in ethylene production in parallel with germination beginning at about 12h of soaking (Figure 1b). In 5 mM ACC detectable levels of ethylene were produced, the matriconditioned seeds producing 10-fold larger than the untreated seeds at the time of germination. It is postulated that seed conditioning greatly increases the ACC-oxidase activity at the time of germination. Germination patterns of untreated or conditioned seeds were similar regardless of soaking in water or in 5 mM ACC indicating that ACC-derived ethylene production is a viable approach for studying germination in seeds with low ethylene producing ability. These data indicate that the soak duration needed is considerably shortened as well as the quantity of ethylene produced greatly enhanced by seed conditioning.

Relief of Thermoinhibition and Enhanced ACC Synthesis and Utilization at High Temperature in Matriconditioned Seeds. Matriconditioning improved the ability of pepper seeds to alleviate high temperature stress. The seeds conditioned at 25 °C emerged earlier than those conditioned at 15 °C (lower T_{50} and higher percent emergence at 5th day), however, the final emergence was reduced markedly in the seeds conditioned at 25 °C (Table 2).

Table 1. Effect of ACC concentrations and soaking time at 25 °C on ethylene production and germination (radicle protrusion) percentage of nonconditioned 'El Paso' pepper seeds

Soaking time (h)	Ethylene (nl/h/100 seeds)			Germination (%)		
	0 mM	1 mM	5 mM	0 mM	1 mM	5 mM
12	0a	0.0a	1.8bc	0a	0.0a	0.0a
24	0a	1.1b	4.2e	0a	0.0a	0.0a
48	0a	1.4b	5.6f	0a	0.0a	0.0a
50	0a	2.3c	5.5f	0a	0.0a	0.0a
52	0a	3.2d	4.9ef	0a	0.0a	2.0a
54	0a	1.3b	2.4c	0a	5.0b	7.5bc
72	0a	1.5b	2.4c	8c	12.5d	19.0e

Mean separation by LSD (P=0.05)

The ACC-oxidase activity of the seeds conditioned at 25 °C increased between 4 to 8h of incubation then declined at 12h. The ACC-oxidase activity of the seeds conditioned at 15 °C, on the other hand, continued to increase during the 12h incubation (Figure 2).

Although the initial level of ACC in pepper seeds conditioned at 25 °C for 4d was lower than in the untreated seeds, and the level decreased further upon transfer to 35 °C, conditioned seeds gradually regained the lost amount and

Table 2. Effect of matriconditioning on viability and vigor of 'El Paso' pepper seeds planted in growth chamber at 35 °C, 12h light/27 °C, 12h dark temperature regime

Matriconditioning (g)	Emergence on 5th d (%)	Emergence on 14th d (%)	T_{50} (d)	Shoot weight of seedlings
Untreated	3a	88b	6.45c	0.82a
4d cond 25C	66c	63a	3.55a	1.21b
7d cond 15C	23b	88b	5.47b	1.07ab

Seeds were matriconditioned in a mixture of 16 g seeds, 4.8 g Micro-Cel E and 16 ml water (containing 0.2% thiram) for 4d at 25 °C and a mixture of 16 g seeds, 4.8 g Micro-Cel E and 22 ml water (containing 0.2% thiram) for 7d at 15 °C in the light in loosely capped glass jars. Matriconditioned seeds were washed and dried by forced air at 25 °C for 2h before sowing in Cornell peat-lite mix soil. Mean separation by LSD (P=0.05)

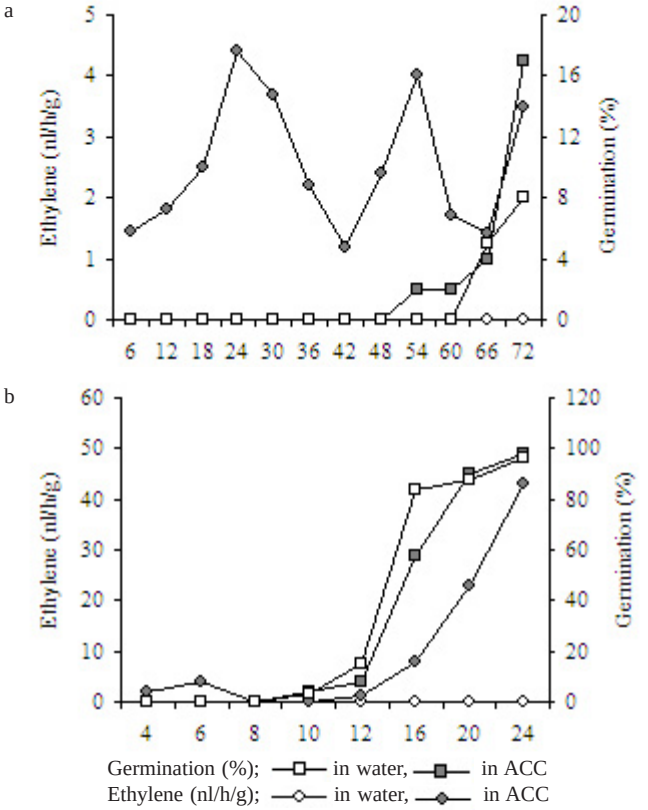

Figure 1. (a) Time course for ACC-derived ethylene production in untreated and (b) matriconditioned 'El Paso' pepper seeds. Untreated or matriconditioned (4d at 25 °C) seeds (0.5 g per replicate, three replicates) were soaked in 5 cm petri plates with 3 ml of 5 mM ACC or water for various time at 25 °C under fluorescent light (9 µmol m^{-2} s^{-1}), and ethylene production determined as described in 'Materials and Methods'. Germination was recorded as radicle protrusion.

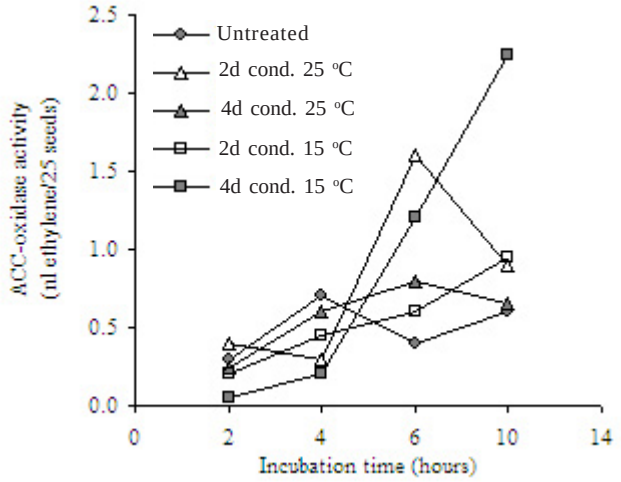

Figure 2. Activity of ACC-oxidase in matriconditioned 'El Paso' pepper seeds incubated in 5 mM ACC at 35 °C.

surpassed the original level during 24h soak (Figure 3a). The ACC level in the untreated seeds, on the other hand, decreased during the 24h soak at 35 °C. Similar pattern was found in the amounts of ACC leaked from untreated and conditioned seeds during the 24h soak at 35 °C (Figure 3b).

DISCUSSION

No ethylene was detected in untreated and conditioned pepper seed in the absence of ACC (Figure 1 & Table 1). When the seeds were soaked in ACC solution, the ethylene producing ability of the untreated seeds increased with increasing concentration of ACC, reaching a measurable level at 5 mM. It has been shown previously in seeds of rice (Khan & Seshu 1986; Khan *et al*. 1987), lettuce, cabbage, tomato, snap bean, and sweet corn (Khan 1994) that a saturating dose of ACC (0.25 to 2 mM depending on the seed type, the amount which the ACC-derived ethylene production is maximum) does not adversely affect germination but increases ethylene production several-fold.

These studies show that the detectable amounts of ethylene produced by untreated pepper seeds by adding ACC in the soak medium provide an effective means to measure ACC-oxidase activity prior to germination (radicle protrusion). In seeds and plant tissues, ACC is the immediate precursor of ethylene and conversion of ACC to ethylene is catalyzed by ACC-oxidase (Yang 1985). Khan (1994) suggest that soaking seeds in ACC solution may have several advantages over seeds soaked in water, i.e. application of ACC increases the ethylene produced by seeds several times over seeds soaked in water, and ethylene detection before germination permits the use of morphologically uniform seed samples.

It was shown that the soak duration needed was considerably shortened as well as the quantity of ethylene produced greatly enhanced by seed conditioning (Figure 1). The ACC-derived ethylene production, which measures ACC-oxidase activity, with several-fold increases in the sensitivity, has been reported previously to be a sensitive measure of seed vigor in rice (Khan & Seshu 1986), lettuce, cabbage,

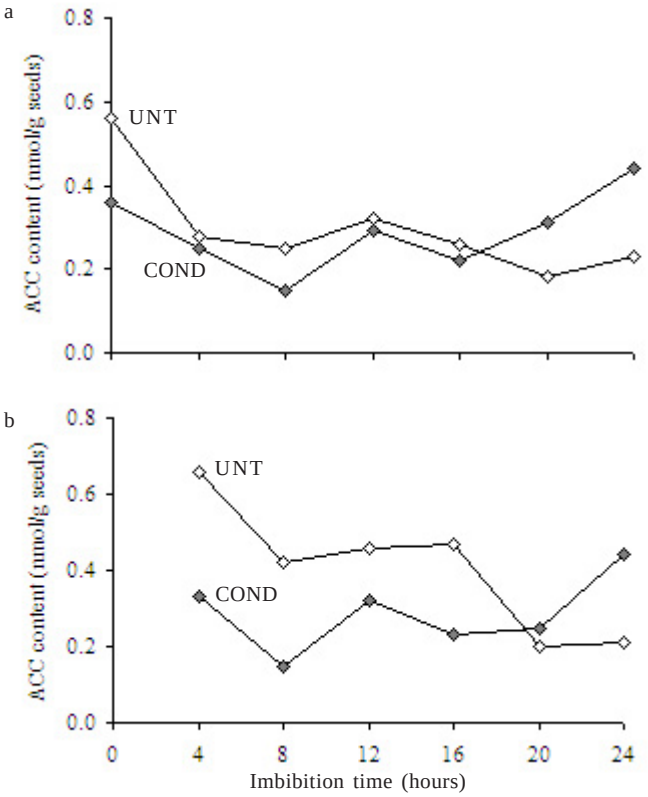

Figure 3. (a) ACC content and (b) ACC leakage in untreated and matriconditioned 'El Paso' pepper seeds imbibed at 35 °C. Seeds were matriconditioned at 25 °C for 4d.

tomato, snap bean, and sweet corn (Khan & Andreoli 1992; Khan 1994). It allows detection of ethylene before germination within a few hours of soaking (Khan 1994). The appearance of two peaks in untreated seeds (Figure 1a) may be related to two distinct ethylene producing events, the first one perhaps related to cellular degradation of the endosperm in preparation for germination and the second one to germination (radicle protrusion). It is well known that injury to cellular tissues lead to increase ethylene production (Yang *et al*. 1985). When germination of chickpea seeds was monitored in distilled water at 25 °C, ACC content and ethylene production from embryonic axes peaked at 24h, which corresponded to a maximum radicle emergence; a decrease in these parameters was observed after radicle protrusion (Gomez-Jimenez *et al*. 2001). A study in seeds of *Striga hermonthica* showed that treatment with 50 mM ACC after 24h imbibition in dark at 33 °C induced the highest percentage germination, equal to that measured for seed stimulated with 10 mM ACC after 7d imbibition in dark at 33 °C, while ethylene treatment induced a lower germination percentage in seeds imbibed for 24h relative to that obtained with 7d imbibition (Logan & Stewart 1995). Application of ACC increased germination of thermosensitive 'Dark Green Boston' lettuce seed at 35 °C to 92% as compared to 5% in water (Kozareva *et al.* 2005). Either ethylene evolution or addition of ACC has been associated with increased activity of endo-β-mannanase, a cell-wall enzyme that weakens the endosperm and allows lettuce seed to germinate at high temperature 35 °C (Nascimento 2003). In conditioned seeds,

endosperm presumably digested during prolonged conditioning. Degradation of endosperm during matriconditioning has been reported in carrot seeds (Dawidowicz-Grzegorzewska & Maguire 1992). The ability of matriconditioned seeds to produce large amounts of ACC-derived ethylene suggests that matriconditioned pepper seeds may have a large pool of ACC-oxidase.

Pepper seeds conditioned at 25 °C showed faster germination than those conditioned at 15 °C, however, the final emergence was reduced markedly in the seeds conditioned at 25 °C (Table 2), and this is coincided with declining of ACC-oxidase activity after 10h incubation in 5 mM ACC in 25 °C conditioned seeds while it still increased in 15 °C conditioned seeds (Figure 2). These data again suggest that 25 °C matriconditioning might adversely influence cellular processes associated with ACC-oxidase activity and its ability to convert ACC to ethylene. Efficient production of ethylene is closely associated with seed vigor as well as the ability to alleviate stress (Huang & Khan 1992; Khan & Andreoli 1992). The data indicate that matriconditioning at 25 °C might predispose the seeds to changes that adversely affect post germination events such as emergence at high temperatures. This is consistent with the poor performance of 25 °C conditioned 'El Paso' pepper seeds observed in field plantings or 'California Wonder' germinated at 32 °C compared to untreated and 15 °C conditioned seeds (Ilyas 1994). Hence, seeds must be conditioned under temperature lower than their optimum temperature for germination. In case of pepper seeds, sowing temperatures for commercial production range from 21 to 28 °C, and germination occurs about one week earlier at 28 than at 21 °C (Smith 1979).

Pepper seeds conditioned for at 25 °C for 4d regained the ACC content after 16 to 20h imbibition in water at 35 °C, and the amount was higher than in the untreated seeds (Figure 3). This indicates that ACC synthesizing ability, probably involving ACC synthase is enhanced during matriconditioning of pepper seeds. Khan (1996) shows that lettuce seeds matriconditioned with moist solid carrier Micro-Cel E at 15 °C for 20 h allow the seeds to germinate at 35 °C. In addition, germination of chickpea seeds was inhibited at supraoptimal temperatures (30-35 °C), and thermoinhibition was reversed by the application of ethylene or ACC (Matilla 2000).

Matriconditioning improved the ability of pepper seeds to alleviate high temperature stress. The seeds performed well at high temperature and at the same time synthesized ACC and had high ACC-oxidase activity. Thus, the basis of thermotolerance in matriconditioned pepper seeds may be not only the ability to synthesize ACC but also to effectively utilize the endogenously produced ACC. Enhanced synthesis and utilization of ACC as a means to alleviate thermoinhibition has been reported in conditioned lettuce seeds (Khan & Prusinski 1989; Huang & Khan 1992).

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